**Yeast Strain Identification Strategy:** We designed a next-generation sequencing strategy to identify strains present in the diploid yeast knockout library. Sequencing libraries were constructed in two sequential PCR reactions, yielding amplicons with three variable regions: Barcode #1 (eight base pair well location identifier plus a random 12 base-pair unique molecular identifier), the 20 base pair UPTAG sequence (corresponding to mutant strain) and Barcode #2 (eight base pair source plate identifier) (Figure S1A). PCR #1 amplified the UPTAG sequence flanking the KANMX locus of each yeast strain while incorporating Barcode #1. PCR #2 attached adapter sequences necessary for binding and amplification in Illumina sequencing technology, while simultaneously incorporating Barcode #2. Combining Barcode #1 with the UPTAG allowed us to uniquely identify strains and their plate locations. Barcode #2 permitted deconvolution of PCR duplicates from genomic counts. All primers used in this study have been included as a supplemental table (Table S1).

**Yeast Library Maintenance and Library Preparation:** Strains were grown at 30C in 96-well format in liquid YPAD with G418 selection. Crude genomic DNA was extracted via zymolyase digestion of cultures diluted in PBS. 10µL PCR #1 reactions (per well, GBioSciences 786-449)) were performed using crude genomic DNA extract and primers as discussed above. PCR #11 products were pooled by plate, column-purified (NEB T1030L) and bead-cleaned (Beckman Coulter A63881) at an 0.8:1 ratio to remove primer dimer. Primer dimer removal was assessed via BioAnalyzer; bead cleaning was repeated if necessary. Amplicons were normalized by concentration (dsDNA HS Qubit Assay, Thermo Fisher, Q32851). 10uL PCR-2 reactions were performed (per yeast library plate, KapaHiFi HotStart ReadyMix, KK2602) using NEBNext® Multiplex Oligos for Illumina sets 1 and 2 (E7335L and E7500L). Column purification, bead cleaning and BioAnalyzer quality control were repeated; products were quantified. In most cases, primer dimer was significantly reduced or eliminated. PCR #2 products were pooled at equimolar amounts and then diluted to 40pM with 10% or 2% PhiX (Illumina, FC-110-3001) in a 150uL mix. iSeq 100 i1 Reagent cartridges (Illumina, 20021533) were thawed at 4C for 48 hours prior to sequencing. Diluted library was loaded into cartridges according to iSeq onscreen instructions and paired-end sequencing was performed over three separate runs of the Illumina iSeq.

**Data Analysis:** A read alignment approach was used to distinguish knockout strains present in each well from the raw sequencing data. First, we constructed a reference sequence for all expected knockout strains in the library by concatenating the following: well-specific barcoded primers (Table S1), the expected UPTAG region, and KANMX sequence common to all strains. The resulting reference sequence comprised approximately 12,000 different possible contigs. Raw sequencing reads were aligned to the custom reference sequence with Bowtie2. Bowtie2 is well-adapted to confront small indels that may have been introduced during library preparation, as well as degenerate reference sequences such as those arising from the barcoding strategy used here. UPTAG sequences were extracted as the subsequence aligning to the expected regions of the reference and were filtered on a Levenshtein distance of less than 2 from the expected guide sequence. Likewise, well-specific barcodes incorporated in PCR1 (denoting strain locations in the library) were extracted from the variable region flanking the common primer sequence. Strain counts were tallied per well location, resulting in normalized frequencies which were used to classify the strain or mixture of strains present in each well.

Most wells (90%) exhibited at least 30 sequencing reads per well (Figure S1B). Wells with <30 were often found to lack yeast growth (not shown). Strain identities determined via sequencing were compared to strain identities annotated by the library distributor. In cases where mismatches occurred, two metrics were considered. Read Proportion (RP) represents the number of reads obtained from a well that can be attributed to a single strain. RP is subject to PCR quality, as primer dimer can contribute non-meaningful reads. Chastity Score (CS) represents the proportion of reads from the top three strains identified in any well that can be attributed to a single strain. CS is robust to PCR quality, as only reads attributable to the top three strains are considered; CS can be considered a measurement of well contamination/mixing. CS and RP cutoffs were used to define which wells exhibited high enough sequencing quality to warrant re-assignment of strain identity annotation in cases where expected and observed annotations differed. For wells with >100 reads, cutoffs of 0.6 for CS and 0.5 for RP were used; for wells with 30 > n > 100 reads, cutoffs of 0.8 for CS and 0.5 for RP were used (Figure S1C). No wells with fewer than 30 reads per well were re-assigned. 316 strain identities were re-assigned as a result of this approach, totaling to 4467 unique strains in the library (Figure S1D).